Delineating Immune-Mediated Mechanisms Underlying Hair Follicle Destruction in the Mouse Mutant Defolliculated

Fiona Ruge[1], Aikaterini Glavini[1], Awen M. Gallimore[2], Hannah E. Richards[2], Christopher P. Thomas[2], Valerie B. O'Donnell[2], Michael P. Philpott[3] and Rebecca M. Porter[1]

Defolliculated (Gsdma3^{Dfl}/+) mice have a hair loss phenotype that involves an aberrant hair cycle, altered sebaceous gland differentiation with reduced sebum production, chronic inflammation, and ultimately the loss of the hair follicle. Hair loss in these mice is similar to that seen in primary cicatricial, or scarring alopecias in which immune targeting of hair follicle stem cells has been proposed as a key factor resulting in permanent hair follicle destruction. In this study we examine the mechanism of hair loss in GsdmA3^{Dfl}/+ mice. Aberrant expression patterns of stem cell markers during the hair cycle, in addition to aberrant behavior of the melanocytes leading to ectopic pigmentation of the hair follicle and epidermis, indicated the stem cell niche was not maintained. An autoimmune mechanism was excluded by crossing the mice with rag1−/− mice. However, large numbers of macrophages and increased expression of ICAM-1 were still present and may be involved either directly or indirectly in the hair loss. Reverse transcriptase-PCR (RT-PCR) and immunohistochemistry of sebaceous gland differentiation markers revealed reduced peroxisome proliferator-activated receptor-γ (PPARγ), a potential cause of reduced sebum production, as well as the potential involvement of the innate immune system in the hair loss. As reduced PPARγ expression has recently been implicated as a cause for lichen planopilaris, these mice may be useful for testing therapies.

Journal of Investigative Dermatology (2011) **131,** 572–579; doi:10.1038/jid.2010.379; published online 16 December 2010

INTRODUCTION

Cicatricial alopecias, a group of hair loss disorders in humans that involve the total destruction of the hair follicle (HF) with associated inflammation, are also referred to as scarring alopecias because of the replacement of the HF with connective tissue (Somani and Bergfeld, 2008). In some, such as lichen planopilaris (LPP), the sebaceous gland appears atrophic because of either inflammation targeting the gland or abnormal function. Defolliculated (Gsdma3^{Dfl}/+) mice are one of several mouse mutations where a defective sebaceous gland is associated with hair loss with similarities to scarring alopecia. Gsdma3^{Dfl}/+ share a similar phenotype to other mice with a mutation in the *gasdermin A3* (*Gsdma3*) gene and to Asebia (Scd1^{ab2J}) mice that have a defective stearoyl CoA desaturase 1 (Scd1), an enzyme important in lipogenesis in both the sebaceous gland and adipose tissue (Zheng *et al.*, 1999; Sundberg *et al.*, 2000; Runkel *et al.*, 2004; Lunny *et al.*, 2005; Tanaka *et al.*, 2007). Similarities include hair loss beginning at 4 weeks, HF destruction (8–15 weeks), chronic inflammation, and reduced lipid synthesis in the sebaceous gland. In addition, the HF undergoes a defective catagen, failing to complete regression (Porter *et al.*, 2002).

A number of hypotheses have been put forward to explain the mechanism of HF destruction in cicatricial alopecias. These include: (1) An autoimmune mechanism that not only targets the HF but specifically damages or eliminates stem cells of the bulge as a result of a collapse in immune privilege (Karnik *et al.*, 2009; Harries *et al.*, 2010). (2) Interference in dermal–epidermal signaling by the immune system. The HF requires intricate epithelial–mesenchymal communication between the dermal papilla and the stem cell niche to form a follicle and to control the hair cycle. Hence, the immune system may not necessarily interfere by physically damaging cells but by producing signaling molecules that disrupt this communication (McElwee, 2008). (3) Reduced sebaceous

[1]*Department of Dermatology and Wound Healing, School of Medicine, Cardiff University, Cardiff, UK;* [2]*Department of Medical Biochemistry and Immunology, School of Medicine, Cardiff University, Cardiff, UK and* [3]*Centre for Cutaneous Research, Barts and The London, Queen Mary's School of Medicine and Dentistry, London, UK*

Correspondence: Rebecca M. Porter, Department of Dermatology and Wound Healing, School of Medicine, Cardiff University, Cardiff, CF14 4XN, UK. E-mail: rebeccaportersw@yahoo.co.uk

Abbreviations: Gsdma3, gasdermin A3*; HETE, hydroxyeicosatetraenoic acid; HF, hair follicle; LPP, lichen planopilaris; PPAR, peroxisome proliferator-activated receptor; RT-PCR, reverse transcriptase-PCR; Scd1, stearoyl CoA desaturase 1*

Received 8 March 2010; revised 26 October 2010; accepted 1 November 2010; published online 16 December 2010

gland differentiation leading to reduced sebum synthesis. This is believed to cause friction in the upper follicle so that, rather than the hair shaft growing out of the follicle, the fiber is forced backward frequently, leading to penetration and damage of the outer root sheath (Sundberg *et al.*, 2000). (4) More recently, it has been shown that reduced expression of peroxisome proliferator-activated receptor-γ (PPARγ), a nuclear receptor (that acts as a transcription factor on ligand binding), and altered lipid metabolism and peroxisome biogenesis result in scarring alopecia (Harries and Paus, 2009; Karnik *et al.*, 2009; Evers *et al.*, 2010; Stenn and Karnik, 2010). Moreover, these data showing a strong link between lipid metabolism and scarring alopecia have also led to the suggestion that alterations in lipid metabolism may impact signaling. Altered lipid modification of proteins such as hedgehogs and Wnts, which have a fundamental role in HF homeostasis, may be a mechanism by which epithelial–mesenchymal signaling is disrupted (McElwee, 2008; Stenn and Karnik, 2010). The aim of this study was therefore, to test these hypotheses in GsdmA3^{Dfl}/+ mice, thereby determining the potential of these mechanisms for hair loss in humans.

RESULTS

Hair loss is not due to an autoimmune response in Gsdma3^{Dfl}/+ mice

The increased numbers of T cells suggested that an autoimmune response could be a potential mechanism for HF destruction in Gsdma3^{Dfl}/+ mice (Porter *et al.*, 2002). CD3, a marker for all T cells, was immunolocalized to the epidermis and infundibulum, as were γδ T cells. This did not change throughout the morphogenesis and destruction of the HF. CD4, a marker for helper and regulatory T cells and some innate immune cells, was prevalent in the dermis with a few cells in the HF infundibulum and epidermis (Figure 1). CD8-positive cells were not evident.

To investigate whether an autoimmune mechanism is responsible for the phenotype, Gsdma3^{Dfl}/+ mice were crossed onto rag1−/− mice that have a defect in the recombinase-activating gene 1 leading to defective maturation of T- and B-cell lymphocytes (Alt *et al.*, 1992). Gross appearance and histology compared at 8–15 weeks revealed no differences in the timing or degree of HF destruction in Gsdma3^{Dfl}/+ rag1−/− and Gsdma3^{Dfl}/+ rag1+/−, indicating that T and B cells had no role in this process (Supplementary Figure S1 online).

The stem cell niche is not maintained in aberrantly cycling HFs

Previously, we reported that Gsdma3^{Dfl}/+ HFs undergo an aberrant catagen stage where the follicle fails to regress (Porter *et al.*, 2002). This occurs at 17 days and at 7 weeks, as summarized in the diagram in Figure 2. In this study we report that at 8 weeks, the mutant mouse follicles re-enter a second synchronized anagen phase. In contrast, in wild-type mice the hair cycle is no longer synchronized with HFs entering an extended telogen phase. HFs remain in the anagen phase as they are eliminated with follicles that resist destruction longest becoming aberrantly large (Figure 2). Disruption of mesenchymal–epidermal communication is

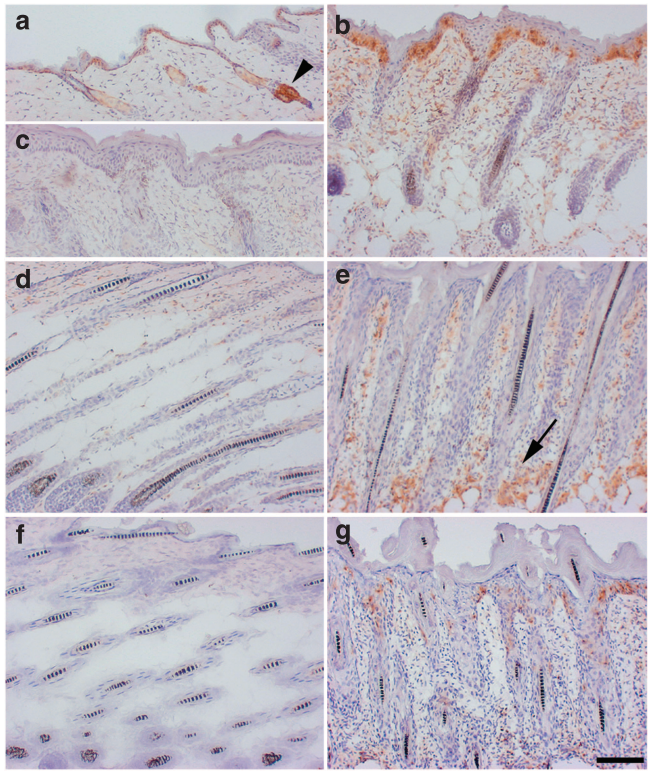

Figure 1. T-cell subtypes in Gsdma3^{Dfl}/+ mice. (**a**) The CD3 antibody gave nonspecific reaction with the bulge (arrowhead) of the hair follicle (HF) in wild-type mice as it was also observed in T-cell-deficient mice (not shown) but (**b**) showed specific reactivity with T-cells in the epidermis and infundibulum of the HF of Gsdma3^{Dfl}/+ mice that (**c**) was depleted when mice were crossed onto rag1−/− background. (**d**) CD4-positive cells were infrequent in wild-type mice and (**e**) found in large numbers, predominantly at the dermal–subcutaneous boundary (arrow) with a few scattered cells in the infundibulum of Gsdma3^{Dfl}/+ mice. (**f**) γδ T cells were absent in wild-type mice and (**g**) immunolocalized to the epidermis and infundibulum in Gsdma3^{Dfl}/+ mice. Scale bar = 100 μm.

highly possible in GsdmA3^{Dfl}/+ mice, as during catagen, the dermal papilla and bulge stem cell niche fail to come into close proximity. As there is evidence that the immune system may have a role in the hair cycle (Paus *et al.*, 1998) and cytokines may promote growth or inhibit apoptosis of keratinocytes (Kemeny *et al.*, 1994; Ruckert *et al.*, 2000), Gsdma3^{Dfl}/+ rag1−/− mice were also examined at 3–7 weeks. Again, the phenotype of lymphocyte-deficient mice was identical to Gsdma3^{Dfl}/+ rag1−/+ mice (Supplementary Figure S1 online), indicating that T cells have no part in the aberrant hair cycling.

Immunohistochemistry was carried out with a panel of bulge stem cell markers Tcf3, Sox9, and CD34 (DasGupta and Fuchs, 1999; Trempus *et al.*, 2003; Vidal *et al.*, 2005). In wild-type mice, immunolocalization of CD34 to the membranes of the bulge region was observed, but never at any time from 3 to 8 weeks in Gsdma3^{Dfl}/+ mice (Figure 3a and b). Tcf3 was located in the nuclei of the bulge of wild-type mice during telogen (Figure 3c) and also in the outer layer of the

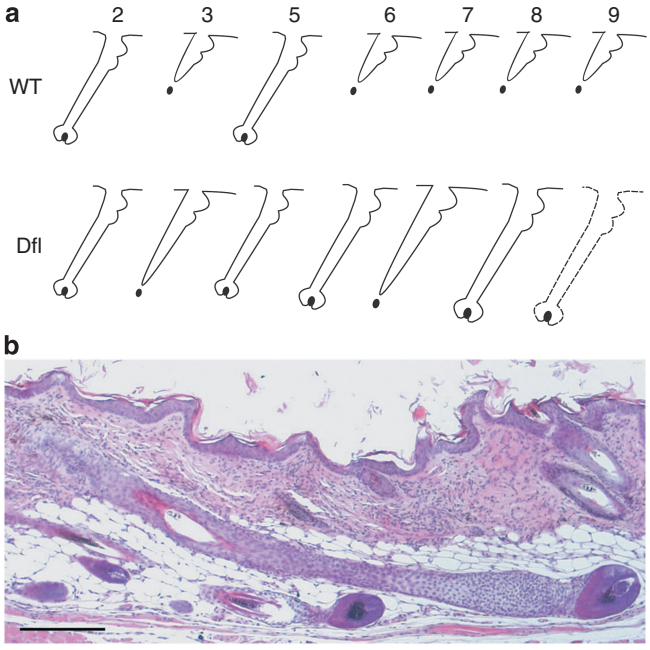

Figure 2. Stages of the hair cycle in Gsdma3^{Dfl}/+ compared with wild-type (WT) mice. (**a**) Diagram to compare the hair cycle of Gsdma3^{Dfl}/+ mice with WT C57Bl/6 mice (2–9 weeks). Gsdma3^{Dfl}/+ mice undergo two aberrant catagen stages at 17 days and 7 weeks. Anagen and the second catagen are slightly delayed compared with WT mice. The hair follicles (HFs) enter a synchronized second anagen at 8 weeks, during which HF deletion takes place. (**b**) At 10 weeks, HFs that have not been deleted can become aberrantly large in Gsdma3^{Dfl}/+. Scale bar = 200 μm.

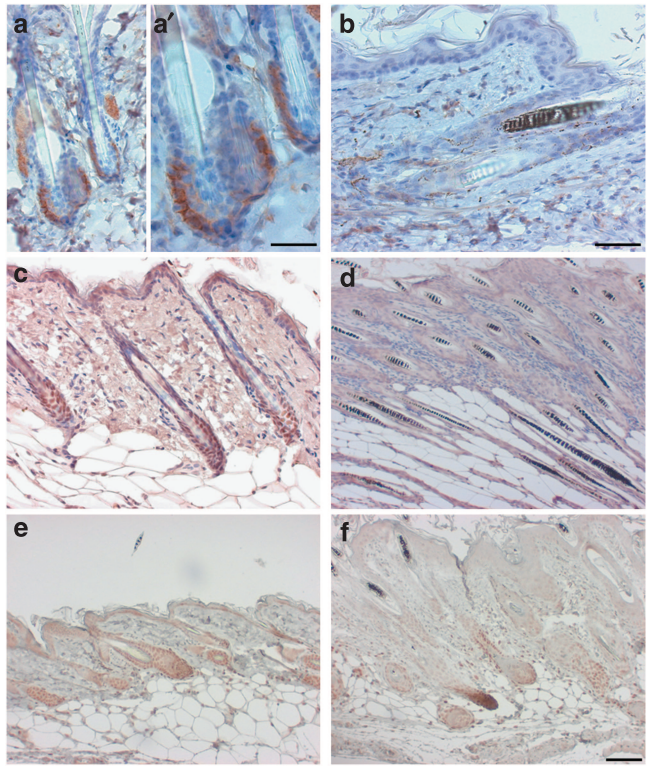

Figure 3. Stem cell markers are absent in Gsdma3^{Dfl}/+. (**a**) CD34 immunolocalized to the hair follicle (HF) bulge in wild-type (**a′** at higher magnification) follicles but (**b**) no immunolocalization to the upper follicle was observed in Dfl/+ rag1−/− mice. (**c**) In telogen, Tcf3 is located in the bulge of wild-type mice but (**d**) is lost from HFs of Gsdma3^{Dfl}/+ mice during the aberrant catagen. (**e**) At 4 weeks, follicles re-enter anagen in wild-type and show Tcf3 in the bulge, but (**f**) in Gsdma3^{Dfl}/+ mice the Tcf3 is restricted to the proximal follicle close to the dermal papilla. Scale bars = 50 μm in **a**–**c**; 20 μm in **a′**; and 100 μm in **d**–**f**.

outer root sheath in cells below the bulge in anagen (not shown) as seen previously (DasGupta and Fuchs, 1999). In Gsdma3^{Dfl}/+ mice, Tcf3 was expressed normally in the outer root sheath during anagen but expression was completely lost from all regions of the catagen HF (Figure 3d). At 4 weeks, as the Gsdma3^{Dfl}/+ HFs are entering anagen, Tcf3 is seen in the proximal follicle close to the dermal papilla rather than the bulge (Figure 3f). Results with the Sox9 antibody were similar to Tcf3 (not shown).

We also examined the melanocytes in Gsdma3^{Dfl}/+ mice and observed that melanin, rather than being deposited solely in the hair shaft, was in all the layers of the epidermis immediately above the HF, in the outer and inner root sheaths and in the dermal papilla (Figure 4). Ectopic tyrosinase (an enzyme involved in melanin production) activity in the outer root sheath and epidermis in Gsdma3^{Dfl}/+ was also detected, beginning during the first hair cycle. Immunolocalization of melanocytes using Pax-3 as a marker for both stem cells and mature melanocytes (Hornyak *et al.*, 2001; Lang *et al.*, 2005; Osawa *et al.*, 2005) suggested that this was due, in part, to migration of melanocytes into the distal as well as in the proximal follicle (Figure 4g).

Reduced sebocyte differentiation in GsdmA3^{Dfl}/+ mice

Reduced sebum production is believed to be the cause of hair shafts failing to exit the follicle smoothly, leading to damage to the HF and chronic inflammation (Sundberg *et al.*, 2000). If this is the case, then the preputial gland, which is also affected by reduced sebum production, should be devoid of inflammation. Comparing the preputial gland of wild-type and Gsdma3^{Dfl}/+ mice by immunohistochemistry with the immune cell markers MAC-1 and CD3, we observed infiltration of macrophages (Supplementary Figure S2 online) but not T cells.

Reverse transcriptase-PCR (RT-PCR) of sebocyte differentiation markers revealed a decrease in Scd1 and Scd3 in GsdmA3^{Dfl}/+ mouse skin (Figure 5). Immunohistochemistry of Scd1 showed expression in the subcutaneous layer as in wild-type mice but the sebaceous gland rarely showed any immunoreactivity. However, on the rare occasion when Scd1 expression was observed, the sebaceous gland also produced sebum. C/EBPα was unchanged and C/EBPβ appeared to be still present in the nucleus of GsdmA3^{Dfl}/+ sebocytes by immunohistochemistry (not shown). PPARα and PPARγ transcription was reduced at all stages of the hair cycle, and immunohistochemistry confirmed the loss of expression of PPARγ in the sebaceous gland (Figure 5).

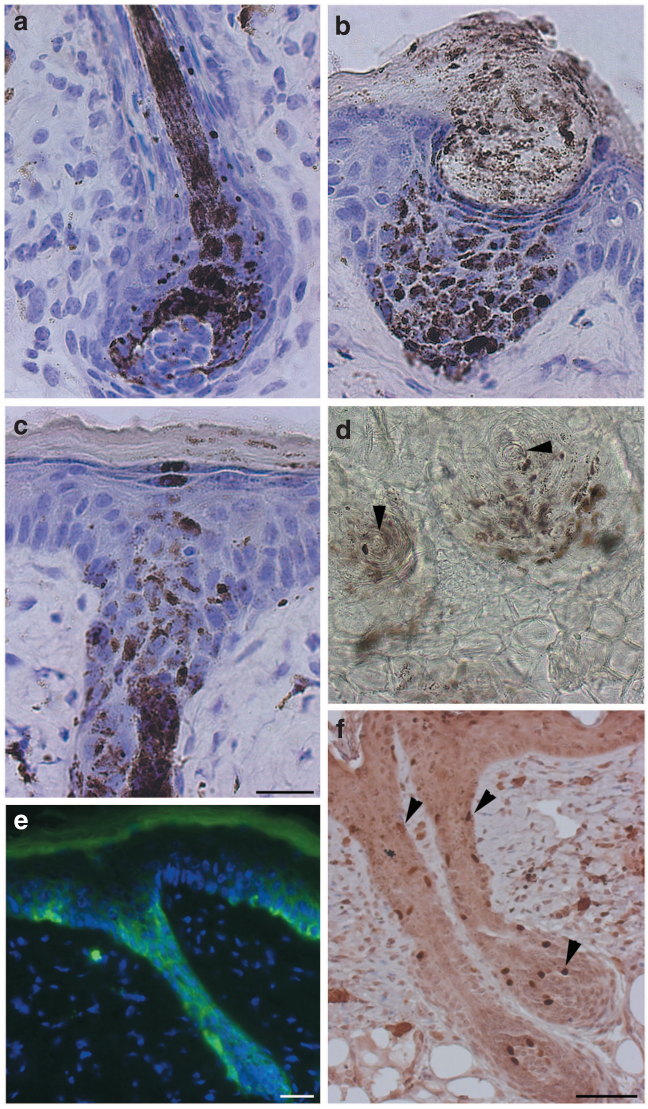

Figure 4. Aberrant location of mature pigment-producing melanocytes. (**a**) Melanin is found in the inner root sheath and hair shaft (**b**) in all layers of the epidermis above the hair follicle (HF) and (**c**) in the outer root sheath. (**d**) From the surface of the skin, melanin is confined to the region surrounding the hair shaft (arrowheads). (**e**) Tyrosinase activity (green) indicates pigment formation in the outer root sheath and epidermis. (**f**) Pax 3-positive nuclei can be seen in the outer root sheath high up in the infundibulum and epidermis (arrowheads) as well as in the bulb. Scale bars = 20 μm in **a**–**c**; 50 μm in **d** and **e**; and 100 μm in **f**.

Chronic inflammation is likely to be because of reduced PPARγ expression in the sebaceous gland

Reduced expression of PPARγ as an underlying cause of LPP is the most recent hypothesis put forward (Karnik *et al.*, 2009). The mechanism proposed is via peroxisome depletion and production of proinflammatory lipids in response to increased 5-lipoxygenase and cyclooxygenase-2 activity. In both LPP and a conditional PPARγ knockout mouse, there is increased activation of macrophages and T cells. The number of macrophages is also extremely high in the dermis of Gsdma3^{Dfl}/+ mice, and at 4 and 8 weeks these were also highly concentrated in the subcutaneous layer (Figure 6). The phenotype was identical when crossed onto the rag1−/− background. However, RT-PCR and western analysis of cyclooxygenase-2 shows no increase in expression in Gsdma3^{Dfl}/+ mice, and RT-PCR of Pex3 does not support a decrease in peroxisome activity (not shown). Analysis of lipids extracted from Gsdma3^{Dfl}/+ mice also showed no increase in arachidonic acid or prostaglandins. However, an increase in 12-hydroxyeicosatetraenoic acid (12-HETE) was observed, suggesting increased 12-lipoxygenase activity (Supplementary Figure S3 online).

In wild-type animals, ICAM-1expression was observed occasionally around HFs as observed previously (Eichmuller *et al.*, 1998), but was upregulated in Gsdma3^{Dfl}/+ rag1+/− and in Gsdma3^{Dfl}/+ rag1−/− mice. This was not solely restricted to HFs but also found strongly expressed in the basal layer of intrafollicular epidermis and in dermal cells (Figure 6c and d).

DISCUSSION

Crossing Gsdma3^{Dfl}/+ with rag1−/− eliminates a lymphocyte-driven autoimmune mechanism but does not rule out an innate immune response. Macrophages were also present in the mice crossed with rag1−/−. Although these macrophages are clearly not being activated by lymphocytes, natural killer cells, which are not depleted, are also capable of activating macrophages in the absence of T cells (Bancroft and Kelly, 1994; Feng *et al.*, 2006), and may have a role in the hair loss phenotype seen in Gsdma3^{Dfl}/+ mice. Programmed organ deletion by macrophages targeting the stem cells has been proposed as a potential mechanism for HF deletion (Eichmuller *et al.*, 1998). Although macrophages are present in Gsdma3^{Dfl}/+ mice, they do not appear to cluster specifically around the bulge but are located throughout the dermis and sometimes in the subcutaneous layer. ICAM-1 expression was also found to be generally expressed around HFs and epidermis rather than clustered in the bulge region, suggesting that macrophages are not targeting the HF specifically. As activated macrophages have multiple roles both in injury and remodeling of tissues (Gordon, 2003), it is difficult to determine the exact role of macrophages in Gsdma3^{Dfl}/+ mice without depleting them. The same can also be said of mast cells that are also activated in these mice (Porter *et al.*, 2002).

The evidence shown here indicates depletion of bulge stem cell markers, but it is not clear when this happens. CD34 is not normally expressed in the bulge until after the first telogen (Blanpain *et al.*, 2004), and Sox9/Tcf3 are found in the proximal follicle during morphogenesis and anagen (DasGupta and Fuchs, 1999; Vidal *et al.*, 2005). Therefore, the loss of these markers in the stem cell niche cannot be monitored before the first catagen/telogen when CD34 expression is switched on and Sox9/Tcf3 become restricted to the bulge. It is not possible, therefore, to be sure if they are depleted by the immune system gradually before this or because of the aberrant hair cycle causing reduced signaling between the dermal papilla and stem cell niche. However, the fact that the pelage HFs are capable of a precocious

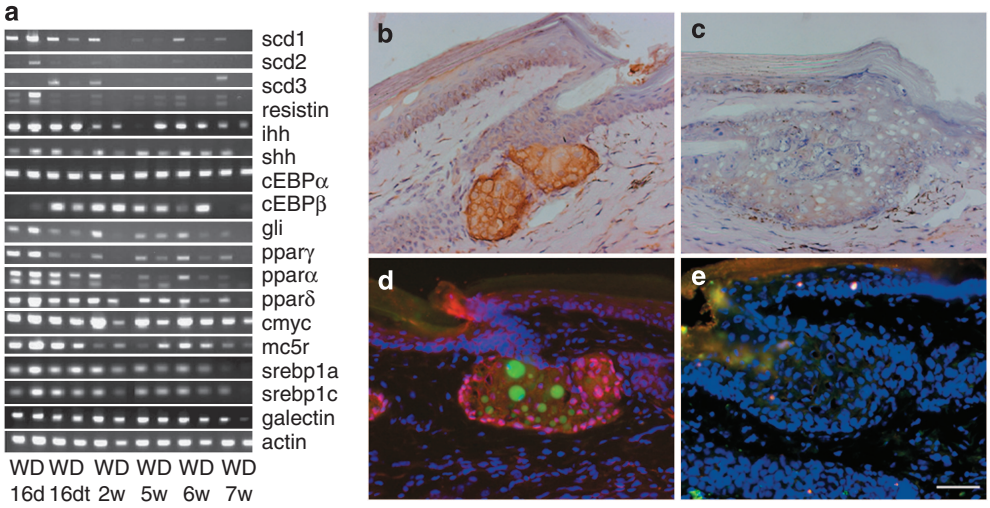

Figure 5. Sebaceous gland differentiation markers. (**a**) Reverse transcriptase-PCR (RT-PCR) of back (or tail) skin was carried out at different stages of the hair cycle and hair follicle (HF) loss. d, days; D, Gsdma3^{Dfl}/+; t, tail; w, weeks; W, wild-type. (**b**) Immunolocalization of stearoyl CoA desaturase 1 (Scd1) is highly expressed in wild-type mice in the center of the gland but (**c**) is absent in most sebaceous glands of Gsdma3^{Dfl}/+ mice. (**d**) Peroxisome proliferator-activated receptor-γ (PPARγ) (red) is present in the nuclei of the outermost cells of the sebaceous gland in wild-type but (**e**) not in Gsdma3^{Dfl}/+ mice. Green indicates intracellular lipids. Scale bar = 50 μm.

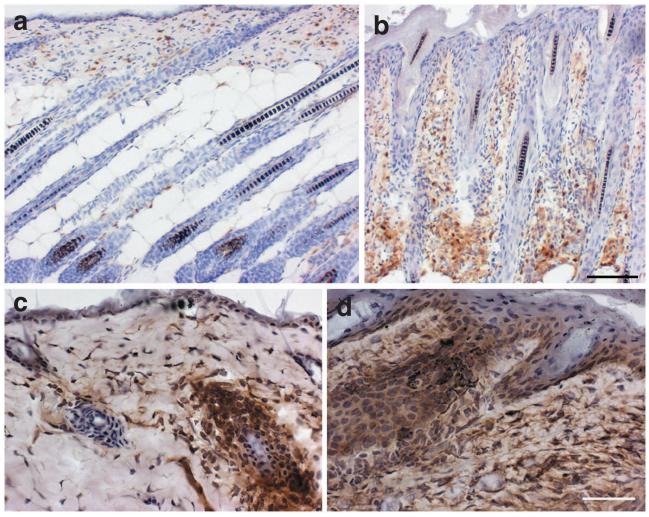

Figure 6. Increase in macrophages in the skin. (**a**) In wild-type animals, occasional macrophages were observed but (**b**) in Gsdma3^{Dfl}/+ mice macrophages were frequently observed in large numbers in the dermis. (**c**) ICAM-1 expression was occasionally seen in wild-type mice around the hair follicle (HF) but (**d**) was found around all the HFs in Gsdma3^{Dfl}/+ mice, in the basal epidermis and in the cells of the dermis. Scale bars = 100 μm in **a** and **b** and 50 μm in **c** and **d**.

synchronized anagen at 8 weeks does not imply that they are being subjected to immune damage. If chronic inflammation was the cause of stem cell deletion, then a gradual deletion of Tcf3/CD34-positive cells would be expected rather than a complete absence of Tcf3/CD34 immunoreactivity in the stem cells at 3 weeks seen here.

Reduction in CD34 expression has been associated with a loss of stem cell quiescence and can occur independently of expression of other stem cell markers (Rhee *et al.*, 2006; Kobielak *et al.*, 2007). This is consistent with the inability of the follicles to enter telogen and the precocious anagen at 8 weeks. Tcf3 is a member of the Tcf/Lef family of DNA-binding proteins that bind to β-catenin on Wnt signaling. However, Tcf3 in the bulge appears to have a β-catenin-independent role in inhibiting stem cell differentiation (Nguyen *et al.*, 2006). Therefore, loss of Tcf3 could have serious consequences for maintenance of the HF stem cell niche.

Along with the relatively quiescent bulge, there is also a pool of stem cells in the HF secondary germ that resides proximal to the bulge in close proximity to the dermal papilla in telogen follicles (Panteleyev *et al.*, 2001; Ito *et al.*, 2004). These cells divide first at the beginning of anagen. The HF predetermination hypothesis proposes that these cells are destined to become the inner root sheath and hair shaft of the next anagen follicle and move down in close proximity to the dermal papilla as a ''lateral disc'' (Panteleyev *et al.*, 2001). The outer root sheath meanwhile is proposed to be derived from the bulge stem cells. Although not all HF labeling experiments support this hypothesis (Legue *et al.*, 2010), there may be some truth in the persistence of hair germ-derived cells residing in close proximity to the dermal papilla, as Tcf3 expression returns to cells in the proximal follicle first at the beginning of anagen in Gsdma3^{Dfl}/+ mice.

Normally, the melanocyte stem cells divide at the beginning of anagen and progeny (melanoblasts) migrate from the bulge down the outer root sheath, only differentiating into mature melanocytes to pigment the developing hair shaft when in close proximity to the dermal papilla (Slominski *et al.*, 2005). The aberrant migration and ectopic pigmentation by the melanocytes in Gsdma3^{Dfl}/+ may therefore also be evidence that catagen/telogen is essential for maintaining

the undifferentiated state of cells in the stem cell niche. However, pigment transfer and localization of melanocytes has been shown to be controlled by the epithelial cells (Weiner *et al.*, 2007; Aubin-Houzelstein *et al.*, 2008).

There is some controversy regarding the role of PPARγ in lipid synthesis in the sebaceous gland. In monolayer cultures of sebocytes, PPAR agonists stimulate lipogenesis (Trivedi *et al.*, 2006), whereas treatment of the intact gland inhibits sebum production (Downie *et al.*, 2004). Scd1 has been shown to contain a PPAR response element in its promoter (Miller and Ntambi, 1996), and hence the lack of PPARγ and Scd1 in the sebaceous glands of Gsdma3^{Dfl}/+ mice does point toward a role for PPARγ in promoting lipogenesis in the sebaceous gland. However, PPARγ depletion in the HF stem cells (Karnik *et al.*, 2009) leads to atrophic sebaceous glands rather than abnormal differentiation as observed in Gsdma3^{Dfl}/+ mice. Although PPARγ is ablated in the stem cells, the effect of PPARγ ablation is unlikely to be on the stem cells themselves, as PPARγ has been shown to be inhibited in the bulge by Tcf3 (Nguyen *et al.*, 2006). Moreover, it is not clear if PPARγ is also depleted in the sebaceous gland by the methods used by Karnik *et al.* (2009).

Increased macrophage accumulation and hair loss have been observed in PPARγ-deficient mice (Karnik *et al.*, 2009) and in pups nursed by PPARγ-deficient mothers because of proinflammatory lipids produced in the milk (Wan *et al.*, 2007). In both these cases, and in LPP, the inflammation seems to be because of proinflammatory lipids produced from arachidonic acid by cyclooxygenase-2, which is not the case in Gsdma3^{Dfl}/+ mice. Although this suggests that Gsdma3^{Dfl}/+ mice are not a model for LPP, there was some patient–patient variation reported when the differentially expressed genes identified by microarray analysis of 20 pooled LPP cases were compared individually (Karnik *et al.*, 2009). There may therefore be several mechanisms for LPP, and lipid analysis of patients should include 12-HETE. The macrophage infiltration into the preputial gland in GsdmA3^{Dfl}/+ strongly points toward dysregulation of lipid synthesis as the underlying cause of chronic inflammation, as damage by the hair shaft can be excluded in this tissue.

PPARγ is expressed in the outermost dividing cells of the sebaceous gland, whereas GsdmA3 is expressed in the most differentiated cells at the center. This suggests communication between differentiated cells and dividing cells. Indian hedgehog is expressed in the most differentiated cells and has been shown to control proliferation but not differentiation of basal cells by a paracrine mechanism (Niemann *et al.*, 2003). However, no reduction in expression of Indian hedgehog in GsdmA3^{Dfl}/+ mice was observed. GsdmA3 may be capable of affecting the differentiation of the basal cells in a paracrine manner or may cause other factor(s) to be released during the holocrine secretion of lipids. As the PPARγ ligand is believed to be a fatty acid, the latter is perhaps more likely.

In conclusion, the phenotype of GsdmA3^{Dfl}/+ mice shares many aspects in common to LPP, including reduced sebum production, chronic inflammation, scarring alopecia, and, as shown here, a reduced expression of PPARγ. What is not clear is whether the aberrant catagen stage is also likely to be a common characteristic with LPP. Whereas in mice the hair cycle is short and synchronized, the human scalp HFs have a hair cycle that is asynchronous with an anagen stage that takes 6–8 years compared with a catagen stage that takes weeks (Saitoh *et al.*, 1970). Therefore, the majority of follicles are in anagen and a defect in catagen could be overlooked. Mutations in GsdmA3 are unlikely to be the underlying cause of LPP, as the incidence of LPP is higher in women and the GsdmA3^{Dfl} mutation affects both sexes equally. However, these mice have a useful role because, unlike the recently generated model for LPP with ablation of the *PPARγ* gene, they are capable of responding to treatment that restores PPARγ activity. Although stimulation with PPARγ agonists is counterintuitive when the level of expression of PPARγ is low or absent, Mirmirani and Karnik (2009) have successfully treated one LPP patient with pioglitazone. Reversal of the phenotype in GsdmA3^{Dfl}/+ mice would prove that PPARγ agonists can restore PPARγ activity despite low levels of expression, and would strengthen the argument for clinical trials for their use in the treatment of LPP as well as providing a model for testing future-generation drugs.

MATERIALS AND METHODS

Mice

Defolliculated (original symbol: Dfl) mice are heterozygous for a spontaneous mutation in the *Gsdma3* gene and are therefore designated the symbol Gsdma3^{Dfl}/+ (Porter *et al.*, 2002; Lunny *et al.*, 2005). Mice were crossed with C57BL/6J (Harlan, UK, Loughborough, UK) to generate Gsdma3^{Dfl}/+ and wild-type controls and housed in scantainers to maintain specific pathogen-free conditions. Rag1−/− mice have a defect in the recombinase-activating gene-1 leading to defective V(D)J recombination (Alt *et al.*, 1992), and are therefore immune compromised, requiring breeding in an isolator. First round of breeding generated Gsdma3^{Dfl}/+ rag1+/− mice identified by their hairless phenotype. These were then bred again onto rag1−/− mice to generate all genotypes including Gsdma3^{Dfl}/+ rag1−/− mice. Rag1−/− mice were identified by the absence of T cells either by flow cytometry using fluorescently labeled antibodies TCR-PE, CD4-PE-Cy3, and CD8-APC (BD-Pharmingen, Oxford, UK) or by immunohistochemistry of spleen sections (see below). All mouse studies were approved by the Cardiff University ethical review panel and in compliance with UK Home Office regulations.

RT-PCR analysis of sebocyte differentiation markers

Complementary DNA was synthesized from RNA (1 μg) extracted with Trizol (Invitrogen, Paisley, UK) from full-thickness dorsal or tail skin using AMV reverse transcriptase (Promega, Southampton, UK) and oligo-dT. See Supplementary Table S1 online for PCR primers.

Immunohistochemistry and immunofluorescence

Full-thickness skin was dissected from the upper dorsal surface or tail of GsdmA3^{Dfl}/+ mice and wild-type littermates at time points from 2 to 15 weeks. Preputial glands were collected from male mice (6–8 weeks). Paraffin-embedded samples underwent antigen retrieval. Frozen sections (5–10 μm) were fixed in dried acetone or 4% paraformaldehyde (15 minutes). Primary antibodies (dilutions in Supplementary Table S2 online) were incubated for 1 hour or

overnight. Biotinylated secondary antibodies (1:200; GE Healthcare UK, Little Chalfort, UK) and streptavidin-conjugated horseradish peroxidase (1:100; GE Healthcare UK) were incubated for 30 minutes at room temperature. Immmunofluorescence was carried out using Alexa Fluor 594 (1:500; Invitrogen) and intracellular lipids were labeled with Bodipy 493/503 (Invitrogen) 1:500 of a 1 mg ml^{-1} stock in ethanol. The tyrosinase assay was performed as described (Han *et al.*, 2002).

Lipid extraction and HETE quantification using liquid chromatography/tandem mass spectrometry

Full-thickness mouse skin was homogenized under liquid nitrogen in the presence of butylatedhydroxytoluene and diethylenetriamine-pentacetic acid to prevent artifactual oxidation. The homogenate was then extracted using hexane/iso-propanol/acetic acid. The extracted lipids were then evaporated to dryness and re-suspended in 100 µl methanol before liquid chromatography/tandem mass spectrometry analysis. Samples were separated on a C18 Spherisorb ODS2, 5 µm, 150 × 4.6 mm column (Waters, Herts, UK) using a gradient of 50–90% B over 10 minutes (A, water/acetonitrile/acetic acid, 75:25:0.1; B, methanol/acetonitrile/acetic acid, 60:40:0.1) with a flow rate of 1 ml min^{-1}. Products were quantitated by liquid chromatography/electrospray ionization/tandem mass spectrometry on an Applied Biosystems 4000 Q-Trap using parent to daughter transitions of *m/z* 319.2 (HETE, $[M\text{-}H]^-$) to *m/z* 179 (12-HETE), 115 (5-HETE), 155 (8-HETE), 167 (11-HETE), 219 (15-HETE), and *m/z* 327 to 184 for 12-HETE-d8, with collision energies of −20 to −30 V. Products were identified and quantified using HETE positional isomers and 12-HETE-d_8 standards run in parallel.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

This work was funded by the National Alopecia Areata Foundation (RM Porter and A Gallimore). A Glavini was funded by the British Skin Foundation (RM Porter, MP Philpott, and Paul E. Bowden, Cardiff University). VB O'Donnell and CP Thomas were funded by the Wellcome Trust. We thank Professor Desmond Tobin for advice on melanocyte markers.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

REFERENCES

Alt FW, Rathbun G, Oltz E *et al.* (1992) Function and control of recombination-activating gene activity. *Ann NY Acad Sci* 651: 277–94

Aubin-Houzelstein G, Djian-Zaouche J, Bernex F *et al.* (2008) Melanoblasts' proper location and timed differentiation depend on Notch/RBP-J signaling in postnatal hair follicles. *J Invest Dermatol* 128:2686–95

Bancroft GJ, Kelly JP (1994) Macrophage activation and innate resistance to infection in SCID mice. *Immunobiology* 191:424–31

Blanpain C, Lowry WE, Geoghegan A *et al.* (2004) Self-renewal, multipotency, and the existence of two cell populations within an epithelial stem cell niche. *Cell* 118:635–48

DasGupta R, Fuchs E (1999) Multiple roles for activated LEF/TCF transcription complexes during hair follicle development and differentiation. *Development* 126:4557–68

Downie MM, Sanders DA, Maier LM *et al.* (2004) Peroxisome proliferator-activated receptor and farnesoid X receptor ligands differentially regulate sebaceous differentiation in human sebaceous gland organ cultures in vitro. *Br J Dermatol* 151:766–75

Eichmuller S, van der Veen C, Moll I *et al.* (1998) Clusters of perifollicular macrophages in normal murine skin: physiological degeneration of selected hair follicles by programmed organ deletion. *J Histochem Cytochem* 46:361–70

Evers BM, Farooqi MS, Shelton JM *et al.* (2010) Hair growth defects in Insig-deficient mice caused by cholesterol precursor accumulation and reversed by simvastatin. *J Invest Dermatol* 130:1237–48

Feng CG, Kaviratne M, Rothfuchs AG *et al.* (2006) NK cell-derived IFN-gamma differentially regulates innate resistance and neutrophil response in T cell-deficient hosts infected with Mycobacterium tuberculosis. *J Immunol* 177:7086–93

Gordon S (2003) Alternative activation of macrophages. *Nat Rev Immunol* 3:23–35

Han R, Baden HP, Brissette JL *et al.* (2002) Redefining the skin's pigmentary system with a novel tyrosinase assay. *Pigment Cell Res* 15:290–7

Harries MJ, Meyer KC, Chaudhry IH *et al.* (2010) Does collapse of immune privilege in the hair-follicle bulge play a role in the pathogenesis of primary cicatricial alopecia? *Clin Exp Dermatol* 35:637–44

Harries MJ, Paus R (2009) Scarring alopecia and the PPAR-gamma connection. *J Invest Dermatol* 129:1066–70

Hornyak TJ, Hayes DJ, Chiu LY *et al.* (2001) Transcription factors in melanocyte development: distinct roles for Pax-3 and Mitf. *Mech Dev* 101:47–59

Ito M, Kizawa K, Hamada K *et al.* (2004) Hair follicle stem cells in the lower bulge form the secondary germ, a biochemically distinct but functionally equivalent progenitor cell population, at the termination of catagen. *Differentiation* 72:548–57

Karnik P, Tekeste Z, McCormick TS *et al.* (2009) Hair follicle stem cell-specific PPARgamma deletion causes scarring alopecia. *J Invest Dermatol* 129:1243–57

Kemeny L, Ruzicka T, Dobozy A *et al.* (1994) Role of interleukin-8 receptor in skin. *Int Arch Allergy Immunol* 104:317–22

Kobielak K, Stokes N, de la Cruz J *et al.* (2007) Loss of a quiescent niche but not follicle stem cells in the absence of bone morphogenetic protein signaling. *Proc Natl Acad Sci USA* 104:10063–8

Lang D, Lu MM, Huang L *et al.* (2005) Pax3 functions at a nodal point in melanocyte stem cell differentiation. *Nature* 433:884–7

Legue E, Sequeira I, Nicolas JF (2010) Hair follicle renewal: authentic morphogenesis that depends on a complex progression of stem cell lineages. *Development* 137:569–77

Lunny DP, Weed E, Nolan PM *et al.* (2005) Mutations in gasdermin 3 cause aberrant differentiation of the hair follicle and sebaceous gland. *J Invest Dermatol* 124:615–21

McElwee KJ (2008) Etiology of cicatricial alopecias: a basic science point of view. *Dermatol Ther* 21:212–20

Miller CW, Ntambi JM (1996) Peroxisome proliferators induce mouse liver stearoyl-CoA desaturase 1 gene expression. *Proc Natl Acad Sci USA* 93:9443–8

Mirmirani P, Karnik P (2009) Lichen planopilaris treated with a peroxisome proliferator-activated receptor gamma agonist. *Arch Dermatol* 145:1363–6

Nguyen H, Rendl M, Fuchs E (2006) Tcf3 governs stem cell features and represses cell fate determination in skin. *Cell* 127:171–83

Niemann C, Unden AB, Lyle S *et al.* (2003) Indian hedgehog and beta-catenin signaling: role in the sebaceous lineage of normal and neoplastic mammalian epidermis. *Proc Natl Acad Sci USA* 100(Suppl 1):11873–80

Osawa M, Egawa G, Mak SS *et al.* (2005) Molecular characterization of melanocyte stem cells in their niche. *Development* 132:5589–99

Panteleyev AA, Jahoda CA, Christiano AM (2001) Hair follicle predetermination. *J Cell Sci* 114:3419–31

Paus R, van der Veen C, Eichmuller S *et al.* (1998) Generation and cyclic remodeling of the hair follicle immune system in mice. *J Invest Dermatol* 111:7–18

Porter RM, Jahoda CA, Lunny DP *et al.* (2002) Defolliculated (Dfl): a dominant mouse mutation leading to poor sebaceous gland differentiation and total elimination of pelage follicles. *J Invest Dermatol* 119:32–7

Rhee H, Polak L, Fuchs E (2006) Lhx2 maintains stem cell character in hair follicles. *Science* 312:1946–9

Ruckert R, Asadullah K, Seifert M *et al.* (2000) Inhibition of keratinocyte apoptosis by IL-15: a new parameter in the pathogenesis of psoriasis? *J Immunol* 165:2240–50

Runkel F, Marquardt A, Stoeger C *et al.* (2004) The dominant alopecia phenotypes Bareskin, Rex-denuded, and Reduced Coat 2 are caused by mutations in gasdermin 3. *Genomics* 84:824–35

Saitoh M, Uzuka M, Sakamoto M (1970) Human hair cycle. *J Invest Dermatol* 54:65–81

Slominski A, Wortsman J, Plonka PM *et al.* (2005) Hair follicle pigmentation. *J Invest Dermatol* 124:13–21

Somani N, Bergfeld WF (2008) Cicatricial alopecia: classification and histopathology. *Dermatol Ther* 21:221–37

Stenn KS, Karnik P (2010) Lipids to the top of hair biology. *J Invest Dermatol* 130:1205–7

Sundberg JP, Boggess D, Sundberg BA *et al.* (2000) Asebia-2J (Scd1(ab2J)): a new allele and a model for scarring alopecia. *Am J Pathol* 156: 2067–75

Tanaka S, Tamura M, Aoki A *et al.* (2007) A new Gsdma3 mutation affecting anagen phase of first hair cycle. *Biochem Biophys Res Commun* 359:902–7

Trempus CS, Morris RJ, Bortner CD *et al.* (2003) Enrichment for living murine keratinocytes from the hair follicle bulge with the cell surface marker CD34. *J Invest Dermatol* 120:501–11

Trivedi NR, Cong Z, Nelson AM *et al.* (2006) Peroxisome proliferator-activated receptors increase human sebum production. *J Invest Dermatol* 126:2002–9

Vidal VP, Chaboissier MC, Lutzkendorf S *et al.* (2005) Sox9 is essential for outer root sheath differentiation and the formation of the hair stem cell compartment. *Curr Biol* 15:1340–51

Wan Y, Saghatelian A, Chong LW *et al.* (2007) Maternal PPAR gamma protects nursing neonates by suppressing the production of inflammatory milk. *Genes Dev* 21:1895–908

Weiner L, Han R, Scicchitano BM *et al.* (2007) Dedicated epithelial recipient cells determine pigmentation patterns. *Cell* 130:932–42

Zheng Y, Eilertsen KJ, Ge L *et al.* (1999) Scd1 is expressed in sebaceous glands and is disrupted in the asebia mouse. *Nat Genet* 23:268–70